Award Number: W81XWH-10-1-0497

TITLE: Regulation of Mammary Tumorigenesis and Lipid Biosynthesis by Spot14

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REPORT DATE: October 2012

TYPE OF REPORT: Annual Summary

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

# REPORT DOCUMENTATION PAGE

*Form Approved*
*OMB No. 0704-0188*



| 1. REPORT DATE | 2. REPORT TYPE | 3. DATES COVERED |
|---|---|---|
| 1 October 2012 | Annual Summary | 15-Sept-2011 – 14-Sept-2012 |

**4. TITLE AND SUBTITLE**

Regulation of Mammary Tumor Formation and Lipid Biosynthesis by Spot 14

**5a. CONTRACT NUMBER**
W81XWH-10-1-0497

**5b. GRANT NUMBER**

**5c. PROGRAM ELEMENT NUMBER**

**6. AUTHOR(S)**

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**5d. PROJECT NUMBER**

**5e. TASK NUMBER**

**5f. WORK UNIT NUMBER**

**7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)**

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13001 E 17th PL Bldg 500 W1126
Aurora, CO 80045-2505

**8. PERFORMING ORGANIZATION REPORT NUMBER**

**9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)**
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

**10. SPONSOR/MONITOR'S ACRONYM(S)**

**11. SPONSOR/MONITOR'S REPORT NUMBER(S)**

**12. DISTRIBUTION / AVAILABILITY STATEMENT**
Approved for Public Release; Distribution Unlimited

**13. SUPPLEMENTARY NOTES**

**14. ABSTRACT**

THRSP/Spot14 regulates de novo fatty acid synthesis in the lactating mammary gland and has also been correlated with a poor patient outcome for women with breast cancer. Despite this correlation, studies have not been done to causatively link Spot14 with breast tumorigenesis. We hypothesized that Spot14 overexpression in mammary epithelial cells that express the Neu oncogene would elevate de novo fatty acid synthesis, resulting in tumor formation, growth, and metastasis. To address this hypothesis, we generated transgenic mice expressing Neu and Spot14 (Neu/S14) in the mammary gland and compared them to Neu controls. Neu/S14 mice develop tumors with a shorter latency and with a higher proliferative index than Neu controls. The tumors, however, are not metastatic. Gene expression profiling revealed an increase in the levels of genes associated with lactation in the Neu/S14 tumors. GC-mas s spectrometry and NMR metabolite studies showed that Neu/S14 tumors have higher lactose and fatty acid levels than Neu tumors. Overexpression of Spot14 in cultured mammary epithelial cells stimulated proliferation but did not stimulate differentiation. We propose a model that predicts Spot14 is expressed in differentiated cells in the mammary epithelium, and that it can stimulate cell proliferation in the presence of oncogenic signaling. These studies indicate that Spot14 might be an important marker of a well-differentiated tumor that is not likely to metastasize.

**15. SUBJECT TERMS**
mammary tumor, breast cancer, Spot 14, lipid synthesis

| 16. SECURITY CLASSIFICATION OF: | | | 17. LIMITATION OF ABSTRACT | 18. NUMBER OF PAGES | 19a. NAME OF RESPONSIBLE PERSON |
|---|---|---|---|---|---|
| a. REPORT | b. ABSTRACT | c. THIS PAGE | | | USAMRMC |
| U | U | U | UU | 27 | 19b. TELEPHONE NUMBER *(include area code)* |

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Introduction:

THRSP/Spot14/S14 is a small cytoplasmic protein that is highly expressed in tissues that synthesize fatty acids de novo. This includes the liver, adipose tissue, and the lactating mammary gland. In the liver, S14 inhibits de novo fatty acid synthesis, but it is required in adipocytes for de novo fatty acid synthesis. S14-null lactating mice produce milk with reduced fatty acid content and nurse offspring with runted development. It has become increasingly clear that tumors display alterations in de novo fatty acid synthesis. Specifically, the enzyme fatty acid synthase (FASN) is often elevated in solid tumor types compared to surrounding normal tissue. High rates of fatty acid synthesis are thought to provide a survival, growth and metastatic advantage to cancer cells. S14 was reported to correlate with a poor patient outcome for women with breast cancer. Based on the role of fatty acid synthesis in cancer, the role of S14 in normal mammary biology, and the effect S14 potentially had on breast cancer outcome, we hypothesized that S14 overexpression in tumors would stimulate fatty acid synthesis, which would promote tumor growth and metastasis. Until this study, nobody had demonstrated a causal link between S14 and mammary tumorigenesis. To establish this link, we have generated a mouse model in which the MMTV promoter drives the mammary specific expression of S14. These mice have been crossed with well-characterized MMTV-Neu mice, which develop mammary tumors with a long latency. In this report, we summarize the studies conducted to date and demonstrate that S14 overexpression in MMTV-Neu mammary glands does indeed promote tumor formation and growth. Interestingly, tumors emerging from S14-transgenic mice are not metastatic. We have extensively profiled these tissues and have developed a proposed model of action for S14, based on the cell type in which we predict it to be expressed. In summary, we suggest that S14 is normally expressed in a well-differentiated cell type in the mammary epithelium, where it stimulates fatty acid synthesis for milk production under the control of prolactin. When prolactin is absent and oncogenic signaling is activated, we predict that the S14-mediated increase in fatty acid synthesis promotes cell proliferation, leading to tumor formation. Because S14 positive cells are well differentiated, the tumors that emerge are not metastatic. This model is supported by our transgenic mouse studies and by analysis of publicly available human breast cancer datasets.

Body:

I have described the studies performed to date in detail below, but to avoid formatting problems, I have chosen to put all of the described figures at the end of the document.

***From SOW: Specific Aim 1****: Determine the effect of Spot14 loss on the growth and metabolism of mammary tumors in vivo in MMTV-Polyomavirus Middle T Antigen (PyMT) mice.*

*Task 1 (Year 2 Months 9-12) We will generate MMTV-PyMT Spot14+/+ (Control) and MMTV-PyMT Spot14-/- (Spot14 null) female mice. These mice will be used for a tumor study, and have an average tumor latency of less than 8 weeks. We expect to generate enough females for our tumor study by the end of the second year.*

We have started breeding male MMTV-PyMT Spot14-/+ with female Spot14-/- mice to generate female MMTV-PyMT Spot14-/- experimental mice and have also bred MMTV-PyMT control mice. We are currently monitoring tumorigenesis in all experimental and control mice.

*Task 2 (Year 3 Months 1-6) We will monitor Control and Spot14-/- females for tumor formation and will harvest tissue when the tumor reaches 1 cm. Since the MMTV-PyMT model promotes tumor formation by 6 weeks, and most tumors reach 1 cm by 12 weeks, we expect to complete this aim by the middle of year 3.*

We are in the process of monitoring experimental and control mice for tumor formation and are measuring tumor growth. The first MMTV-PyMT Spot14-/- female to develop a tumor displayed a rapid tumor growth rate, and we sacrificed her at 10 weeks of age. When we harvested the tissue, we discovered that the tumor was not solid, but instead was a fluid-filled cyst. She had no other palpable tumors, and whole mount analysis of her mammary glands revealed a severe delay in ductal outgrowth and solid tumor formation compared to age-matched control MMTV-PyMT females (Figure 1). The next MMTV-PyMT Spot14-/- mouse to develop a tumor displayed a severe reduction in tumor growth rate. Tumor growth rates for MMTV-PyMT control mice, and MMTV-PyMT Spot14+/- and MMTV-PyMT Spot14-/- mice are shown in Figure 2. The observations made in experimental mice support our hypothesis that Spot14 is required for tumor formation and growth in MMTV-PyMT mice. Studies are ongoing in this model.

*Task 3 (Year 2 Months 6-9) The preserved tumor samples collected from the recipient mice will be analyzed using qPCR, western blot, MRS, and immunohistochemistry. We anticipate completing this task by month 9 in the 3$^{rd}$ year.*

As we are still collecting samples from control and experimental mice, these studies have not yet been completed. We expect to complete them by the target date set in the SOW (Year 3, Month 9).

*Milestone 1: Completion of Specific Aim 1 and preparation of manuscript for publication.*

Although this was originally stated to be "milestone 1", because we have changed the SOW, we now expect this to be milestone 2, and to be completed by the end of the proposal funding period.

***From SOW: Specific Aim 2:*** *Determine the effect of S14 overexpression on the onset, growth, metastasis and metabolism of mammary tumors arising in the MMTV-ErbB2 mice by generating MMTV-ErbB2, MMTV-Spot14 bitransgenic mice.*

We have completed this Specific Aim, and the results of these studies are described below, under each Task.

*Task 1 (Year 1 Months 1-6): We will breed the MMTV-Spot14 and MMTV-c-ErbB2 mice to generate the single and bi-transgenic offspring that will be used in our studies. We anticipate completing this task by the end of the second quarter of the first year.*

This Task has been completed. We have generated sufficient experimental and control mice to conduct powerful studies. The ErbB2 proto-oncogene is also called "Neu". Herein as we discuss results of the studies, we will refer to the control, MMTV-ErbB2 mice as "Neu" and the MMTV-ErbB2, MMTV-Spot14 bitransgenic mice as Neu/S14. The Neu/S14 mice are the experimental group. The remaining Tasks and Aim from the SOW are listed below. After that, please find the overall presentation of the results from the remainder of the analysis of Neu and Neu/S14 mice.

*Task 2 (Year 1 Month 6-Year 2 Month 6): The MMTV-Spot14, MMTV-c-ErbB2, and MMTV-Spot14/MMTV-c-ErbB2 mice will be monitored for tumor onset and growth. The tumors will be evaluated as described in the project proposal and the animals will be sacrificed when the tumor reaches 0.5 cm in diameter. The tumor and other tissues will be harvested and preserved. We anticipate completing this aim in the second quarter of the second year.*

*Task 3 (Year 3 Months 1-6): The tumor and other tissue samples will be analyzed using qPCR, western blot, MRS, and immunohistochemistry. These analyses will also help us determine the effect of Spot14 on tumor cell metastasis, as lung tissues will be evaluated for the presence of mammary cancer cells. We anticipate completing this task by the second quarter of the third year.*

***From SOW: Specific Aim 3:*** *Determine the changes in the expression metabolic enzymes affected by gain or loss of Spot14 function in mammary tumors using microarray analysis.*

*Task 1 (Year 1 Months 9-12): We will perform cDNA microarray analysis on tumor samples from the xenotransplantation of S14-/- and WT transformed mammary epithelial cells described in specific aim 1. We will also determine which pathways are affected by analyzing changes in the expression of specific genes associated with tumor cell metabolism. We anticipate completing this task by the last quarter of the first year.*

*Task 2 (Year 3 Months 1-12): We will perform cDNA microarray analysis on tumor samples from transgenic mice expressing ErbB2, Spot14, or both ErbB2 and Spot14 in the mammary epithelium. This will allow us to identify changes in the expression of specific metabolic pathways modulated by Spot14 using bioinformatics analysis. We anticipate completing this task and this aim by the last quarter of the third year.*

*Milestone 2: Completion of Specific Aims 2 and 3 and preparation of manuscripts for publication.*

We have monitored tumorigenesis in Neu and Neu/S14 mice and found that Neu/S14 mice develop tumors significantly earlier than Neu control mice. These data are shown in Figure 2A. The log-rank p-value for this comparison is p=0.0034, with a hazard ratio of 0.2645 (Neu versus Neu/S14) and a 95% confidence interval (CI) of 0.1087 to 0.6435. We also quantified tumor multiplicity for Neu and Neu/S14 mice. This data is presented as number of tumors per mouse. Most mice had 1 or 2 tumors each, but some had more. We found no significant differences in tumor multiplicity between Neu and Neu/S14 mice (Figure 2B). These data suggest that Spot14 overexpression is sufficient to promote tumor formation in the presence of Neu oncogene activation, but does not act as an oncogene on its own, to increase tumor multiplicity. In other words, these data do not suggest that Spot14 can promote oncogenic transformation in the absence of Neu activation. These data support our hypothesis that Spot14 overexpression would stimulate tumor formation.

Tumor tissue was analyzed using a variety of methods. We began our studies by analyzing Neu signaling pathway activation in tumors from Neu and Neu/S14 mice. Western blot analysis of phosphorylated and total forms of Neu (ErbB2), ErbB3, Akt, and Erk showed us that kinase signaling was very heterogeneous within tumor groups, and was not different between tumor groups. Therefore, elevated Neu signaling in the established tumor could not explain the shortened tumor latency observed in Neu/S14 mice, compared to Neu controls (Figure 3). Using immunohistochemistry (IHC), we found that Neu/S14 tumors had a significantly higher proliferative index, as measured by quantification of Ki67 staining, than Neu control tumors (Figure 4A and 4B). We also performed IHC analysis of cleaved Caspase-3 on tumors from both groups, which indicates apoptotic cells. We did not observe any differences between Neu and Neu/S14 tumors in cleaved Caspase-3 staining. In fact, many tumors did not have large apoptotic regions, and the images shown in Figure 4C represent apoptotic "hot spots" within the tumors.

Spot14 is known to play a role in regulating de novo fatty acid synthesis in the liver, adipose tissue, and the lactating mammary gland. Therefore, GC-Mass Spectrometry (GCMS) was used to evaluate tumor fatty acid content. We found a near universal increase in the content of many fatty acids, including non-esterified (NEFA, Free) fatty acids, and those contained in triglycerides, diacylglycerols, cholesterol esters, and phospholipid membranes (Total). These data are shown in Figure 5A and 5B. Table 1 lists each fatty acid chain length and saturation,

with the measured amount in each group of tumors (average and SEM) and the p-value for each two-tailed, unpaired t-test. Those fatty acids that were significantly different between groups have a p-value less than 0.05 and are depicted in bold and italics. We used qPCR analysis to evaluate the expression of three de novo fatty acid enzymes in Neu and Neu/S14 tumors. ATP-Citrate Lyase (ACLY), Acetyl-CoA Carboxylase (ACC) and Fatty Acid Synthase (FASN) each play a critical role in providing substrates for the de novo synthesis of fatty acids. We did not find any differences in the expression of these genes between Neu and Neu/S14 tumors, suggesting that elevated enzyme levels do not explain the increase in tumor fatty acid content seen in Neu/S14 mice (Figure 5C, 6D, and 6E). Overall, these data suggest that Spot14 overexpression stimulates the synthesis of fatty acids in tumor cells, possibly by influencing the activity of the FASN enzyme, which is consistent with our hypothesis that Spot14 overexpression would promote de novo fatty acid synthesis in mammary tumors.

Lung tissue was collected from all tumor-bearing animals. To evaluate lung metastases, we cut 5 micrometer sections every 50 micrometers throughout the entire lung block for each animal. These sections were stained with H&E to visualize lung metastases. Dr. Paul Jedlicka, who is a board-certified pathologist in our department, assisted in analyzing the lung tissue for metastatic lesions. We quantified lung metastases by counting the number of mice with lung metastases out of the total number of mice examined (Figure 6A). Dr. Jedlicka also evaluated the primary tumors for signs of peri-tumoral lymphovascular invasion (LVI). Overall, we found that 1/15 Neu/S14 mice had metastasis of tumors to the lung, and we found that no tumors had signs of LVI. Conversely, 6/17 Neu mice had lung metastases. Evaluation of LVI revealed invading cancer cells in 3 Neu primary tumors. Of these 3 tumors, we already observed lung metastases in 2 of them, but 1 tumor had not yet metastasized to the lung at the time of sacrifice. If we combine this data, we found that, overall, 7/18 Neu mice had invasive or metastatic tumors, while this was observed in only 1/15 Neu/S14 mice. The Fisher's Exact p-value for this difference is 0.046, meaning Neu/S14 tumors are significantly less likely to be invasive or metastatic than Neu tumors. These data are reported in Figure 6B, as an embedded table that depicts the differences in metastasis and invasion found between groups. Surprisingly, these data contradicted our hypothesis. We predicted that Spot14 overexpression would stimulate fatty acid synthesis in tumors, which would give them a *growth and metastatic* advantage. What we found, however, was that Spot14 overexpression did stimulate tumor growth, but the Neu/S14 tumors were not highly metastatic.

The reduction in metastatic and invasive ability of Neu/S14 tumors seemed to contradict the increase in tumor cell proliferation observed in this group. To identify novel differences in gene expression, we performed cDNA microarray analysis of Neu (N=13) and Neu/S14 (N=11) tumors. CEL files were produced from the Affymetrix Gene Atlas instrument. These CEL files were RMA normalized and Log2 transformed using Partek Genomic Suite software. Spreadsheets of combined data were then analyzed using Significance Analysis of Microarrays (SAM[1]) in Excel. Rather than using ANOVA, followed by a multiple-testing correction, SAM

includes a false discovery rate (FDR) correction in the initial analysis, and reports data as fold change values (experimental versus control) and gives q-values. The SAM q-value is equivalent to a standard p-value. We created a list of genes that were different between Neu/S14 and Neu tumors at a q-value of 5% (0.05). At this significance level, we found that 456 genes were significantly elevated in Neu/S14 versus Neu tumors, while 26 were decreased. As an aside, I earned my PhD studying lactation in mouse models, so as I looked at the gene list, I noticed that many of the genes elevated in Neu/S14 tumors were also genes that I knew to be associated with the lactating mammary gland. To scientifically approach this, I took advantage of data from a separate ongoing study in our laboratory. For that separate study, we performed microarray analysis on enriched mammary epithelial cells from day 14 pregnant and day 4 lactating mice. I was able to cross-reference the genes elevated in lactation with the genes elevated in the Neu/S14 tumors, and found that 41% (188/456) of the genes increased in Neu/S14 tumors were also increased in the lactating mammary gland. The genes elevated at least 1.5 fold are listed in a table in Figure 7 (Figure 7A), which also includes one gene that was repressed in Neu/S14 versus Neu tumors. The genes listed in bold type are also elevated in lactation, and the genes with an asterisk (*) were validated using qPCR analysis. One of the most significantly elevated genes in Neu/S14 tumors was Elf5 (Figure 7B). Published studies have shown that Elf5 acts as a "master regulator" of mammary alveolar cell expansion during pregnancy and lactation[2]. In fact, mice lacking Elf5 in the mammary gland fail to lactate. Elf5 is thought to act downstream of prolactin signaling during lactation to participate in the induction of differentiation-associated genes during lactation. Additionally, in human breast cancer cells, Elf5 is necessary to mediate the proliferative effects of progesterone[3]. Together, these studies suggest that Elf5 can promote both proliferation and differentiation of the mammary gland. These data were quite surprising, but also explained the decreased metastatic ability of Neu/S14 tumor cells, compared to Neu controls. Lactation represents a time when the mammary epithelium goes through the process of terminal differentiation. Among other significantly elevated genes was the epithelial marker, keratin 18 (Figure 7C). Finally, Elf5 was shown to promote the expansion of a population of alveolar epithelial cells that lacks the cell surface marker, CD61 or B3-integrin (Itgb3). We found that Itgb3 expression in Neu/S14 tumors trends towards being significantly lower than Neu tumors (Figure 7D). Serum prolactin levels have been measured in MMTV-S14 and wild type mice, and have shown that S14 overexpression does not influence the levels of circulating prolactin. Therefore, the effects of S14 on tumor differentiation are likely independent of elevated circulating prolactin (Figure 7E). Differentiated cancer cells are thought to be less metastatic than undifferentiated cells.

One study has been published that demonstrated an increased risk for breast cancer patient death associated with S14 protein levels[4]. Specifically, these investigators found that S14 protein was directly correlated to increasing tumor grade, and that lymph-node positive breast cancers expressing high S14 were more likely to kill the patient than those with low S14. There are several points to make regarding this study. First, the association of S14 with tumor grade and with patient outcome suggests that S14 is not an independent predictor of death from breast

cancer. High tumor grade is known to correlate with reduced survival of breast cancer patients. Second, the investigators analyzed lymph-node positive tumors, which means, to some extent, these cancers were already invasive. Third, the antibody used for IHC analysis of S14 in this study was made by the investigators, and they have not made the antibody available for other investigators to use, so unfortunately, we cannot repeat their study in an independent breast cancer sample set. To determine if S14 gene expression was correlated with patient outcomes for breast cancer, I analyzed publicly available microarray data as an alternative to performing IHC analysis on breast tumor samples. I obtained gene expression datasets of primary human breast tumors, either from Oncomine[5] or the NCBI Gene Expression Omnibus (GEO). Each of these datasets had expression information for S14 (called THRSP on the array platforms), and also had some sort of patient outcome data. This included either disease-free survival, where disease is defined as cancer recurrence or cancer metastasis, or disease-specific survival, which is defined as death from breast cancer, and will be referred to as "overall survival". The details of these datasets are presented in Table 4. Initially, I found the median expression level for S14 in each dataset, and all patients with above median expression were called "high S14" expressing, while those below the median were called "low S14" expressing. This allowed me to convert S14 expression to a categorical variable, which meant the datasets could be combined. When I analyzed the outcomes of these patients using a Kaplan-Meier approach, with a Log-rank (Mantle-Cox) test, I found that there were no differences in patient outcome correlating with S14 expression (data not shown). To more precisely separate the cases into high and low S14 expressing groups, I sorted the datasets individually by S14 expression, and took the upper and lower 25% of the samples to use for analysis, giving me the more extreme high and low expressing tumors. Performing the statistical analysis on these groups, representing "high S14" and "low S14", respectively, showed me that patients with high S14 expressing tumors were significantly less likely to experience disease recurrence or metastasis, and were also significantly less likely to die from disease, compared to patients with low S14 expressing tumors (Figure 8). These data are exciting, and support the observations seen in our mouse model. Together, these data suggest that S14 overexpression is associated with a particular tumor cell type that is well differentiated and is not likely to metastasize. The question remained, then, does S14 promote differentiation, or does is simply permit the expansion of a differentiated cell type within the pre-tumorigenic mammary gland?

To address this question, I used mouse mammary epithelial cells, which were engineered to express S14 under the control of a doxycycline-inducible (dox) promoter. Dox addition results in a dose-dependent increase in S14 expression levels in this model (Figure 9A). S14 was induced for 24 hours, and then cells were monitored for proliferation for an additional 4 days. These cells were compared to cells that were not treated with dox. We found that S14 overexpression in cultured mammary epithelial cells significantly increased proliferation over the course of the 4 day assay, compared to cells without S14 induction (Figure 9B). RNA was harvested from cells with and without S14 expression and qPCR analysis was performed on several differentiation-associated genes that were identified using microarray analysis of mouse

tumors (see Table 3). Analysis of Elf5, Keratin 18, and Butyrophilin 1a1 (Btn1a1) showed that S14 induction did not increase the expression of these genes, known to be associated with mammary epithelial cell differentiation (Figure 10). We also assayed the levels of Cck, Csn1s2a, and Lao1, all of which were increased in Neu/S14 compared to Neu tumors, but these genes were not expressed in CIT3 cells, and showed no increase with S14 induction (data not shown). Together, these data suggest that S14 promotes the proliferation of cells in which it is expressed, but does not promote differentiation of mammary epithelial cells, per se.

Based on these studies, we predicted that S14 overexpression would promote proliferation in the mammary gland of MMTV-Neu mice before tumors form. To determine if this was the case, we performed whole-mount analysis on mammary glands from age-matched (10 months) and diestrus-staged Neu and Neu/S14 females. The mammary gland is known to experience cycles of proliferation in response to the elevated progesterone levels that occur during the estrus cycle (www.mammary.nih.gov). Thus, when analyzing mammary glands from nulliparous, cycling female mice, it is important to monitor the estrus cycle and harvest tissue from females that are in the same stage of their cycles. We found that Neu/S14 mammary glands displayed hallmarks of hyperplastic alveolar nodules (HAN), which were not seen in Neu control glands (Figure 9C). These data suggest that S14 overexpression in MMTV-Neu mice is sufficient to stimulate proliferation of the mammary epithelium.

From these results we have created a model that predicts a role for S14 in breast cancer (Figure 11). In the normal mammary gland, S14 expression increases beginning in late pregnancy and is dramatically elevated in the epithelial compartment during lactation. At this time, the mammary gland is being instructed by prolactin signaling. We predict that S14-mediated fatty acid synthesis supports milk production during lactation, as that is what prolactin is guiding the mammary epithelium to do. Based on the normal mammary gland, we predict that high S14 expression is associated with a well-differentiated epithelial cell type. When S14 is highly expressed in the absence of prolactin, and in the presence of oncogenic signaling, for example, we hypothesize that it stimulates fatty acid synthesis, through a similar mechanism that is seen during lactation, but these cells are not being guided to make milk. Instead, we hypothesize that these fatty acids are signaling to the cell to proliferate, which shortens the process of tumor formation in the presence of the Neu oncogene. This results in the more rapid formation of a tumor, but because a very particular type of cell is proliferating, these tumors are not metastatic. Instead they are well differentiated, and express many genes found in the lactating mammary gland. The results of this study are being prepared for publication, and we anticipate submitting a manuscript to Cancer Research by the end of October.

The remainder of this grant period will be focused on analyzing tumorigenesis in MMTV-PyMT mice with and without S14 expression. Initial studies performed in these mice suggest that S14 expression is required for tumor cell proliferation, which is consistent with results obtained in Neu/S14 bitransgenic mice. We will soon be able to identify if S14 loss causes the depletion of a particular cell type within the PyMT tumors.

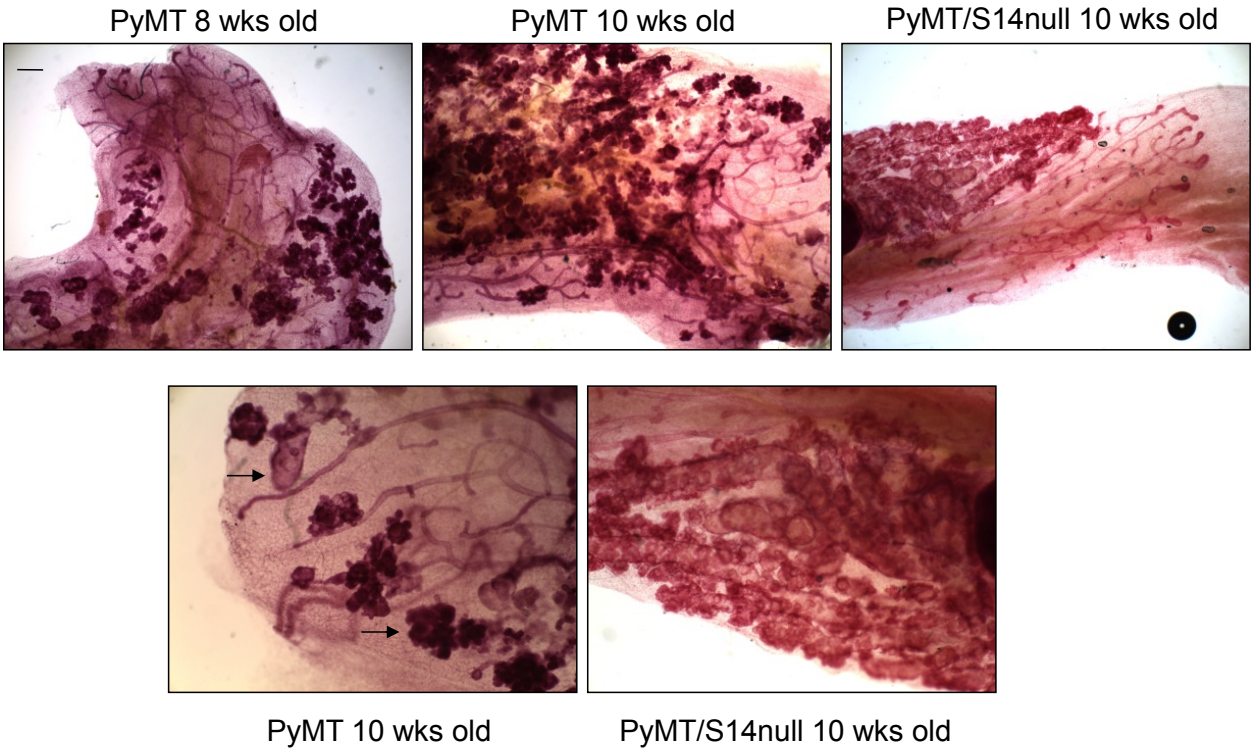



**Figure 1. Whole mount analysis of mammary glands from MMTV-PyMT and MMTV-PyMT/S14-null mice.** Scale bar=500 micrometers

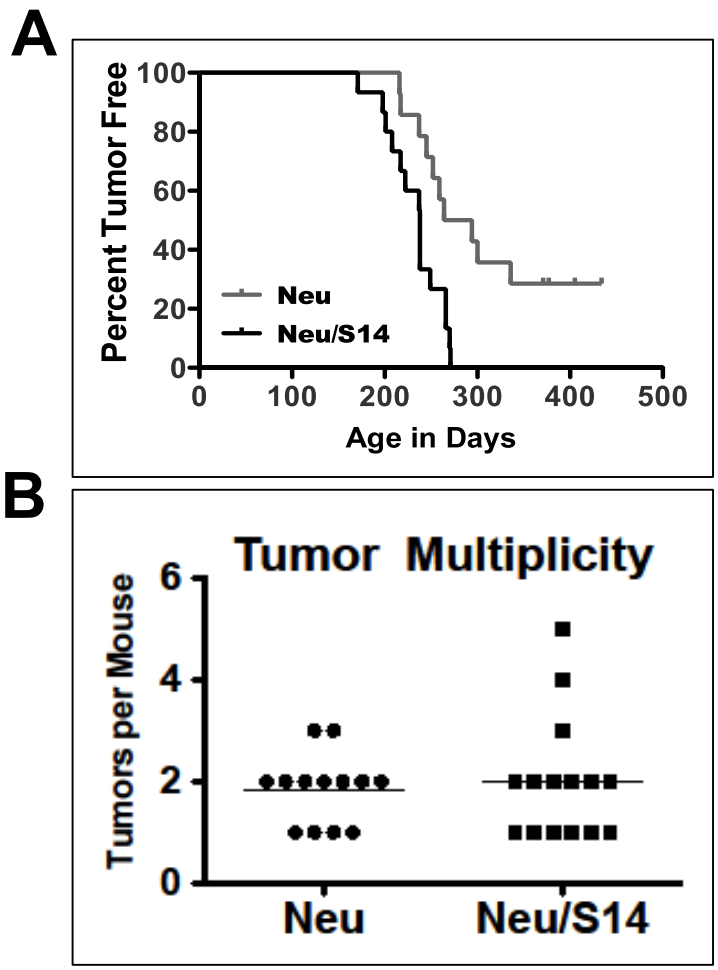



**Figure 2. Analysis of tumorigenesis in Neu and Neu/S14 mice.** A) Kaplan-Meier survival curves of tumor latency in Neu and Neu/S14 mice. Log-rank p-value = 0.0034, showing S14 expression is associated with a significantly shorter tumor latency. B) Tumor multiplicity in Neu and Neu/S14 mice, expressed as number of tumors per mouse.

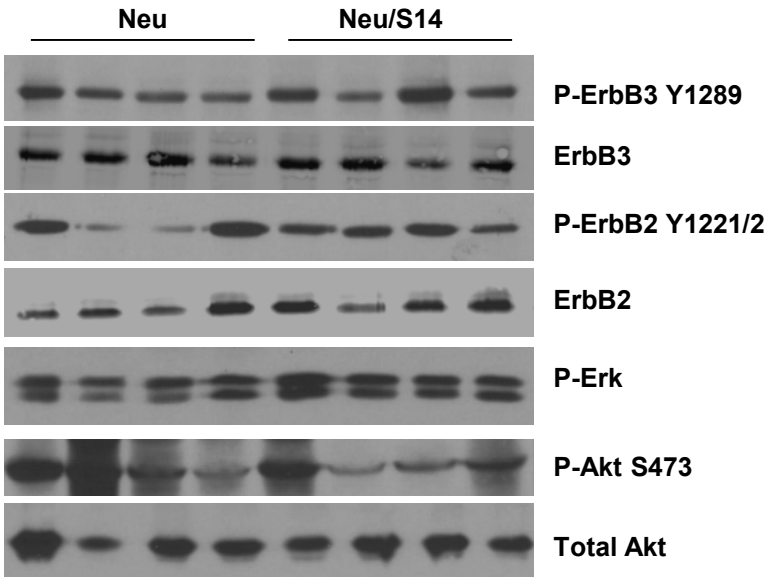



**Figure 3. Western Blot analysis of tumors from Neu and Neu/S14 mice.** Differences were not detected in signaling pathway activation between Neu and Neu/S14 tumors. Signaling molecule activation was highly variable within tumor groups.

| Fatty Acid Chain Length and Saturation | Total Fatty Acids (ng per mg tissue) | | Ratio Neu/S14 to Neu | p-value | Non-esterified Fatty Acids (ng per mg tissue) | | Ratio Neu/S14 to Neu | p-value |
|---|---|---|---|---|---|---|---|---|
| | Neu | Neu/S14 | | | Neu | Neu/S14 | | |
| **10:0** | 0.209 | 0.688 | 3.3 | 0.104 | 0.021 | 0.034 | 1.6 | 0.142 |
| **12:0** | 0.165 | 0.235 | 1.4 | 0.213 | 0.025 | 0.042 | 1.7 | 0.117 |
| **14:0** | 0.635 | 1.153 | 1.8 | ***0.027*** | 0.145 | 0.238 | 1.6 | ***0.004*** |
| **14:1** | 0.029 | 0.098 | 3.4 | ***0.013*** | 0.013 | 0.023 | 1.8 | ***0.002*** |
| **16:0** | 4.248 | 8.308 | 2.0 | ***0.012*** | 0.862 | 1.706 | 2.0 | ***0.003*** |
| **16:1** | 0.479 | 1.257 | 2.4 | ***0.017*** | 0.077 | 0.211 | 2.7 | ***0.003*** |
| **18:0** | 4.026 | 5.240 | 1.3 | 0.099 | 0.814 | 1.260 | 1.5 | ***0.019*** |
| **18:1** | 4.182 | 7.349 | 1.8 | ***0.028*** | 0.721 | 1.619 | 2.2 | ***0.005*** |
| **18:2** | 2.713 | 5.725 | 2.1 | ***0.025*** | 0.421 | 1.056 | 2.5 | ***0.007*** |
| **18:3** | 0.019 | 0.016 | 0.8 | 0.218 | *nd* | *nd* | - | - |
| **20:4** | 4.686 | 3.576 | 0.8 | 0.230 | 0.307 | 0.400 | 1.3 | 0.069 |

**Table 1. GCMS analysis of fatty acids in tumors from Neu and Neu/S14 mice.** The fatty acid chain lengths and saturations, the average content of fatty acids in Neu (N=7) and Neu/S14 (N=7) tumors, and the ratio of each fatty acid average in Neu/S14 versus Neu tumors are listed. Two-tailed t-tests were used to determine if differences between groups were statistically significant. P-values in bold and italics are significant ($p<0.05$).

| Metabolite | Neu | | Neu/S14 | | pvalue |
|---|---|---|---|---|---|
| | Average | SEM | Average | SEM | |
| **Lactose** | **1.31** | **0.23** | **1.99** | **0.23** | ***0.05*** |
| GPC | 2.65 | 0.26 | 2.43 | 0.37 | *0.35* |
| tCholine | 0.82 | 0.19 | 0.50 | 0.06 | *0.10* |
| GSH | 0.34 | 0.21 | 0.10 | 0.01 | *0.18* |
| DMA | 0.25 | 0.06 | 0.17 | 0.02 | *0.15* |
| Glutamate | 2.77 | 0.86 | 3.79 | 0.61 | *0.21* |
| Acetate | 0.26 | 0.07 | 0.28 | 0.05 | *0.42* |
| **OH-Butyrate** | **3.36** | **0.42** | **1.58** | **0.68** | ***0.05*** |
| Acetyl-CoA | 0.28 | 0.12 | 0.24 | 0.06 | *0.42* |
| Val, Leu. Ile | 3.03 | 0.65 | 4.20 | 0.10 | *0.08* |

**Table 2. NMR analysis of aqueous metabolites in tumors from Neu and Neu/S14 mice.** Perchloric acid was used to extract aqueous metabolites from frozen tumor tissue. The amounts of each metabolite are per mg of tumor tissue. The only significant differences between groups were observed in lactose and OH-butyrate. Lactose is a milk disaccharide, which is produced by differentiated alveolar epithelial cells. Student's unpaired two-tailed t-tests were used to compare metabolite levels between groups.

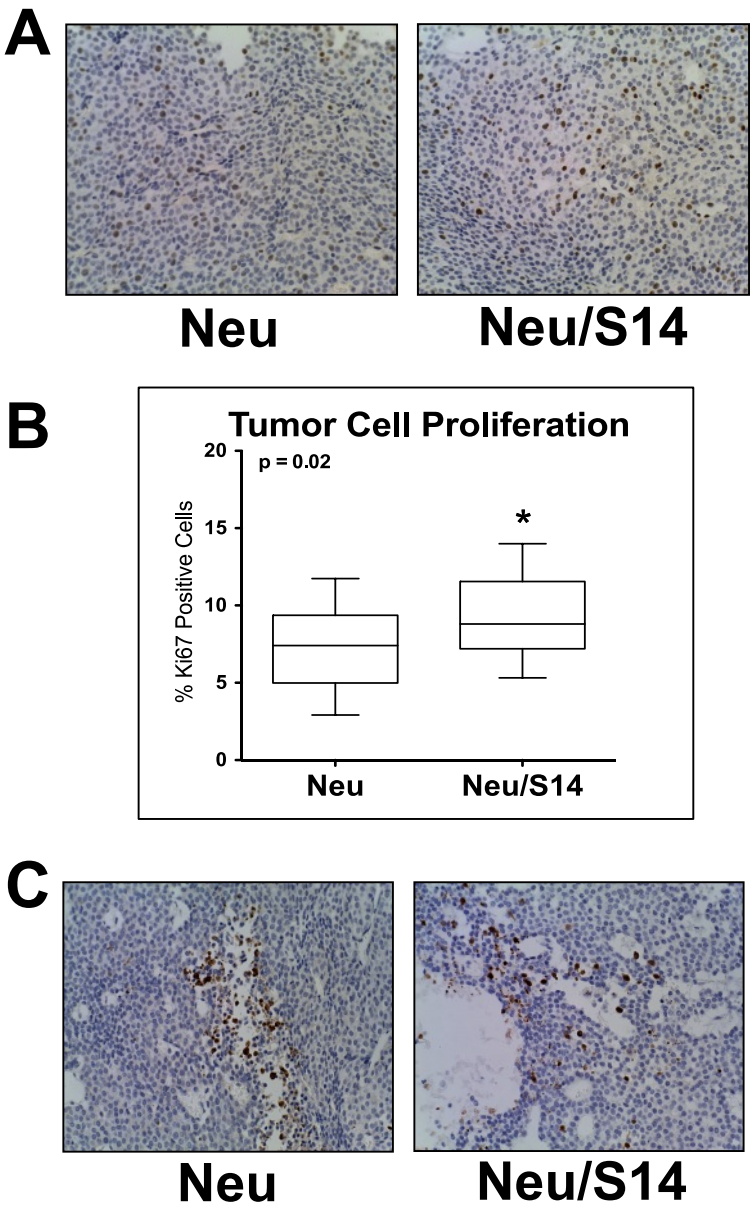



**Figure 4. Analysis of proliferation and apoptosis in Neu and Neu/S14 tumors.** IHC was used to analyze Ki67 (A) in tumors from both groups. The number of Ki67 positive nuclei out of total nuclei per 20X field from 5 images per tumor of 5 tumors per group was counted and is reported in (B). A student's unpaired, two-tailed t-test was used, and showed a pvalue of 0.02 for the difference in proliferation between groups. IHC analysis was also used to evaluate cleaved Caspase-3 (C) as a marker of apoptosis. Overall, apoptosis was low in tumors from both groups and did not appear to be different.

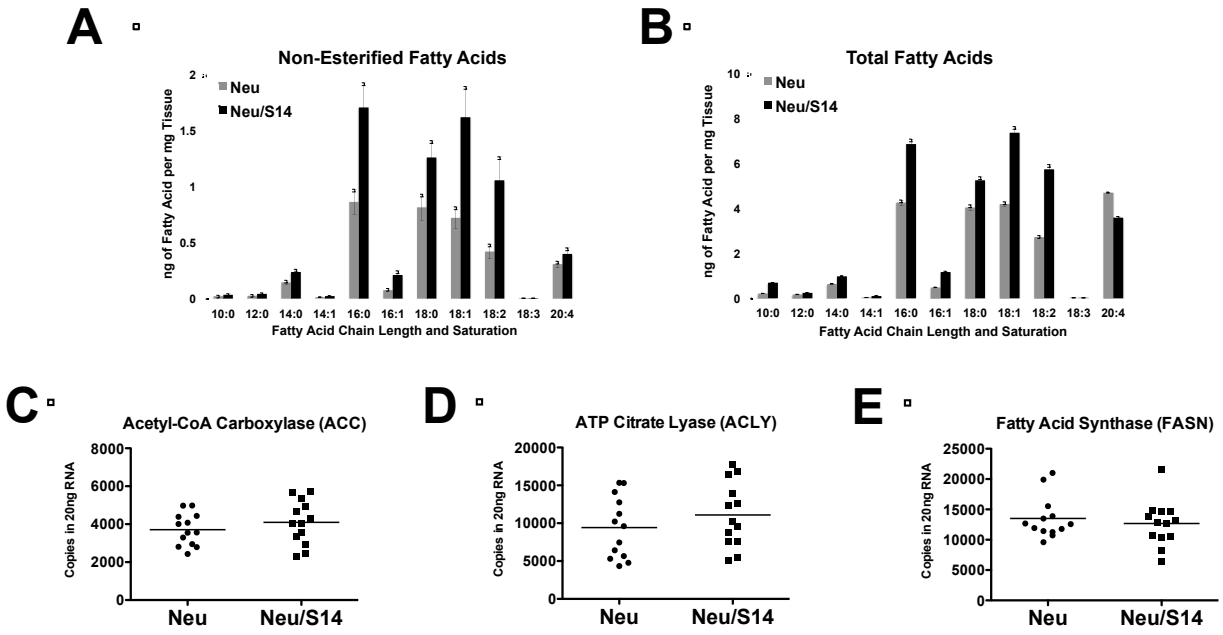
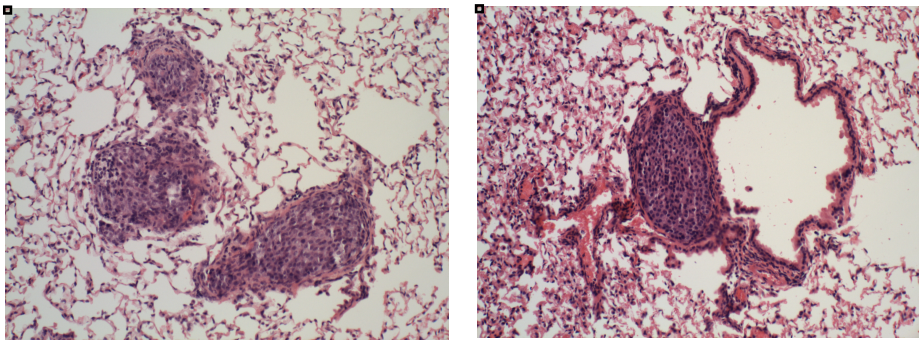



**Figure 5. Analysis of fatty acids and de novo fatty acid synthesis enzymes in Neu and Neu/S14 tumors.** GCMS was used to analyze non-esterified or free fatty acids (A) and total fatty acids (B) in mouse tumors. Nearly all fatty acids analyzed were elevated in Neu/S14 versus Neu tumors. QPCR analysis of de novo fatty acid synthesis pathway enzymes Acetyl-CoA Carboxylase (ACC, C), ATP-Citrate Lyase (ACLY, D), and Fatty Acid Synthase (FASN, E), showing no differences in expression between groups.

A



Neu Neu/S14

B

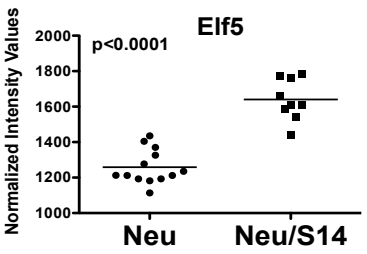
| Group | Invasive or Metastatic/total |
|---|---|
| Neu | 7/18 (38.9%) |
| Neu/S14 | 1/15 (6.7%) |

**Figure 6. Analysis of invasion and metastasis in Neu and Neu/S14 mice.** A) Representative metastatic lesions in the lungs of Neu (left) and Neu/S14 (right) mice. B) Quantification of mice with invasive or metastatic tumors out of total mice examined. Fisher's Exact test was used to determine that S14 overexpression is associated with a significantly reduced risk of invasion and metastasis (p=0.046).

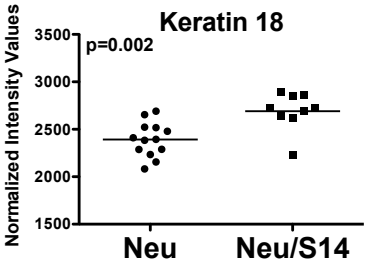
**A**

| Neu/S14 > Neu | |
|---|---|
| **Gene** | **Fold** |
| *** Lao1** | **3.86** |
| *** Csn1s2a** | **3.52** |
| *** Cck** | **3.28** |
| *** Btn1a1** | **2.93** |
| **Prol1** | **2.68** |
| **C87977** | **2.60** |
| *** Arg2** | **2.43** |
| **Cd36** | **2.24** |
| **Cytip** | **2.16** |
| Tomm5 | 2.03 |
| **Oog1** | **1.98** |
| Spink5 | 1.97 |
| LOC100503984 | 1.96 |
| **Olah** | **1.90** |
| Snord61 | 1.89 |
| **Pcdhb17** | **1.87** |
| Rny1 | 1.82 |
| **Gm13023** | **1.81** |
| **Ust** | **1.75** |
| * Lef1 | 1.75 |
| **Sdr16c5** | **1.74** |
| **Mfsd2a** | **1.73** |
| **Dpp4** | **1.72** |
| Entpd1 | 1.65 |
| Slc2a9 | 1.63 |
| Rgs1 | 1.62 |
| **Sectm1a** | **1.61** |
| *** Olfr234** | **1.61** |
| Ada | 1.60 |
| *** Thrsp** | **1.57** |
| **Smr2** | **1.55** |
| **Gulp1** | **1.54** |
| Chn2 | 1.53 |
| Rnasel | 1.53 |
| **Tcfcp2l1** | **1.50** |
| * Cpeb2 | 0.61 |

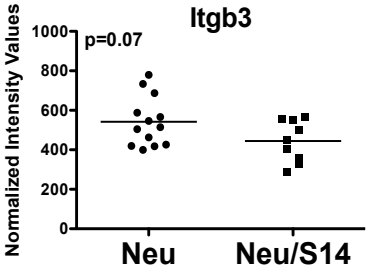
**B**




**C**




**D**




**E**

| Genotype | Prl (ng/mL) |
|---|---|
| Wild Type | 23.6 ± 3.3 |
| MMTV-S14 | 18.4 ± 7.6 |

**Figure 7. Microarray analysis of Neu and Neu/S14 tumors reveals differences in cellular differentiation.** A) Table of genes elevated in Neu/S14 versus Neu tumors greater than 1.5 fold, and including one gene decreased in Neu/S14 versus Neu tumors. Genes in bold are also elevated during lactation, and the asterisk (*) denotes genes whose expression changes were validated using qPCR. Increases in differentiation-associated genes, Elf5 (B) and Keratin 18 (C) were found in Neu/S14 tumors. The cell surface marker, CD61 or B3-integrin (Itgb3) represents an alveolar precursor, and when expression is lost, cells are thought to differentiate (D). E) Serum prolactin levels are not different between MMTV-S14 and wild type mice.

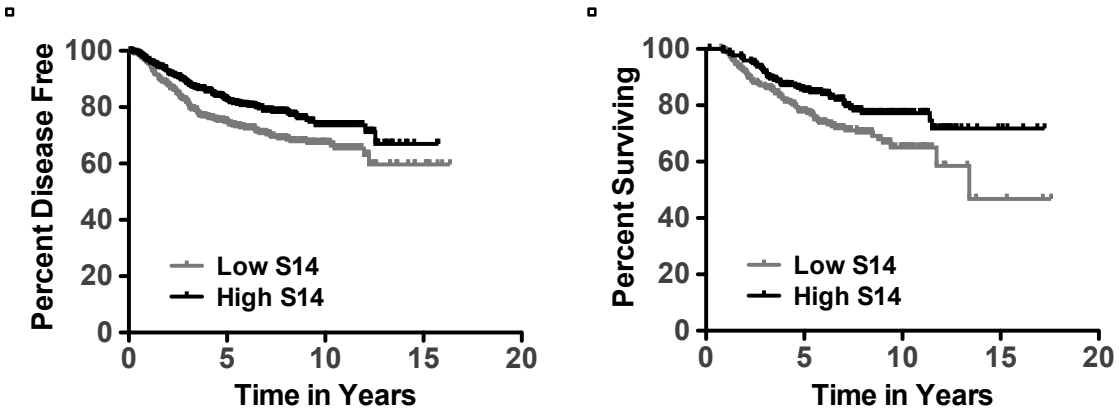



**Figure 8. Kaplan-Meier survival curves from primary human breast cancer data.** Details of analysis are described in the text above. Patients with high S14 expressing tumors are less likely to experience disease recurrence or metastasis (left; Log Rank pvalue 0.0073) or to die from disease (right; Log Rank pvalue 0.013) compared to patients with low S14 expressing tumors.

| GEO ID | Total Tumors | Low S14 | High S14 | Platform | PMID | Analysis Performed |
|---|---|---|---|---|---|---|
| GSE19615 | 115 | 29 | 29 | HG-U133P2 | 20098429 | Disease-Free Survival |
| GSE6532 | 414 | 102 | 91 | HG-U133A, HG-U133B, HG-U133P2 | 20479250, 18498629, 17401012 | Disease-Free Survival |
| GSE20685 | 327 | 82 | 82 | HG-U133P2 | 21501481 | Disease-Free Survival, Overall Survival |
| GSE1456 | 159 | 40 | 40 | HG-U133A, HG-U133B | 16280042 | Disease-Free Survival, Overall Survival |
| N/A (Available from Oncomine) | 295 | 73 | 73 | Printed Microarray | 12490681, 11283592 | Overall Survival |
| GSE4922 | 249 | 63 | 63 | HG-U133A, HG-U133B | 17079448 | Disease-Free Survival |
| GSE21653 | 266 | 60 | 59 | HG-U133P2 | 20490655, 22110708 | Disease-Free Survival |
| GSE22226 | 130 | 33 | 33 | Agilent Human Genome 44K | 22198468 | Disease-Free Survival, Overall Survival |
| **Total Tumors** | 1955 | 482 | 470 | | | |
| Disease-Free | 1660 | 409 | 397 | | | |
| Overall | 911 | 228 | 228 | | | |

**Table 3. Details of datasets used for survival analysis.** Data were obtained from Oncomine or from the NCBI Gene Expression Omnibus (GEO). All data were normalized within datasets. The details of the analysis are described in the above text.

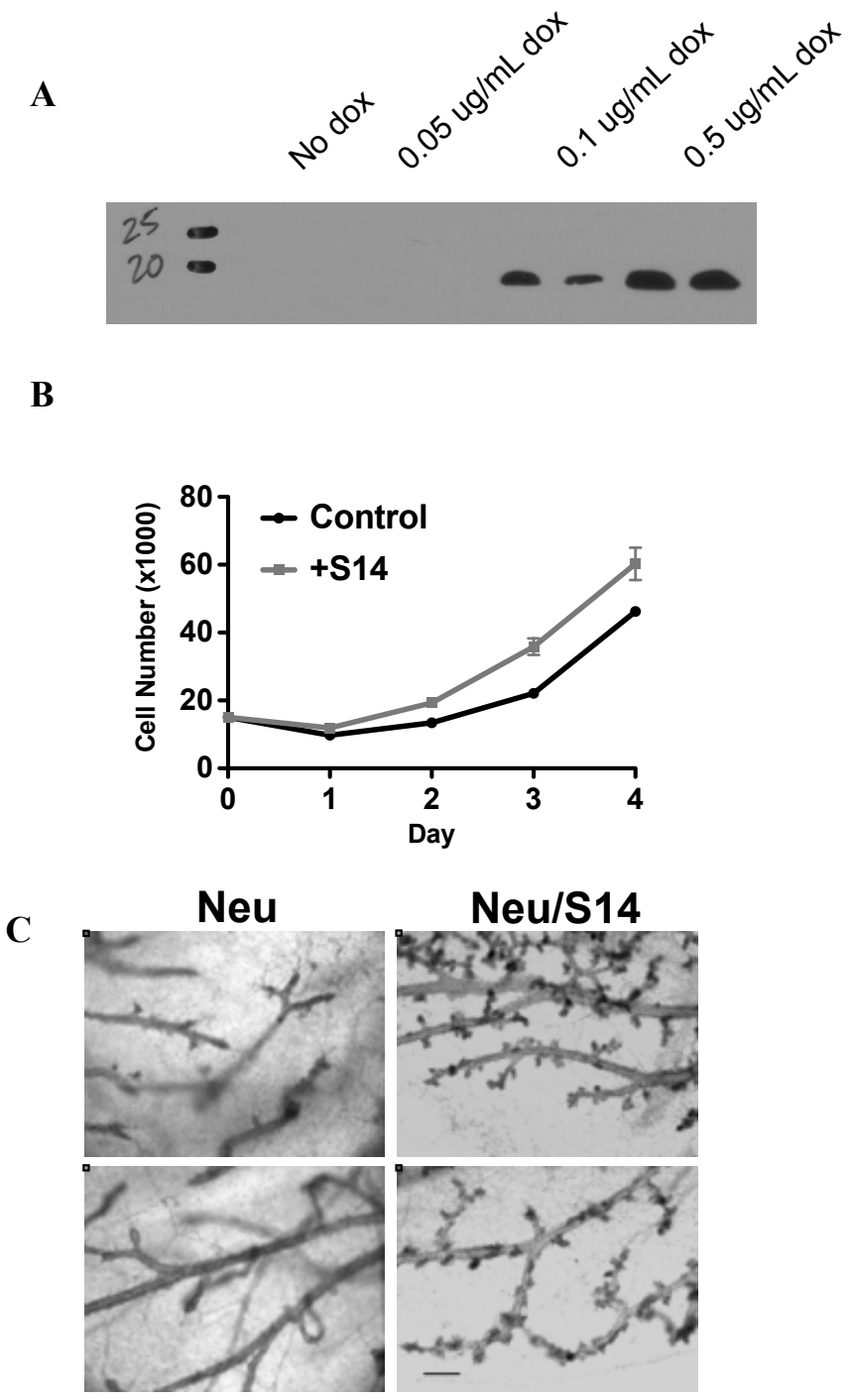



**Figure 9. Analysis of proliferation in normal mammary epithelial cells and non-tumor bearing mammary glands.** A) Western blot analysis of HA protein levels following doxycycline treatment of CIT3 cells. B) Proliferation assay of CIT3 mammary epithelial cells with and without S14 expression. Beginning at day 2, the S14 expressing cells are significantly higher than controls cells. Student's two-tailed t-test was used to compare control versus S14 cells at each day. Days 2-4 pvalue <0.001. C) Whole mount images of mammary glands from Neu and Neu/S14 mice. Note the alveolar nodules present in Neu/S14 glands.

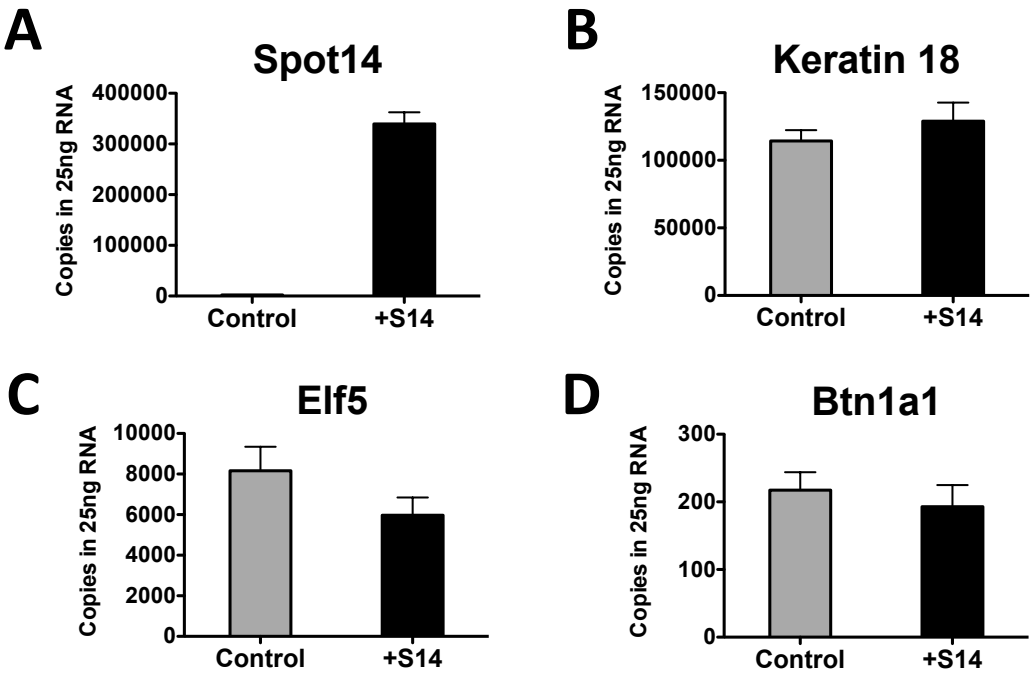



**Figure 10. Q-PCR analysis of differentiation-associated genes in CIT3 cells with and without S14 expression.** CIT3 cells were treated with vehicle or doxycycline, to induce S14, for 48 hours. RNA was isolated and qPCR analysis was performed for Spot14 (A), Keratin 18 (B), Elf5 (C), and Btn1a1 (D). S14 did not stimulate the elevation of differentiation genes in CIT3 cells. Cck, Lao1, and Csn1s2a, three genes that were elevated in Neu/S14 versus Neu tumors, were also analyzed but were not detectable in CIT3 cells (data not shown).

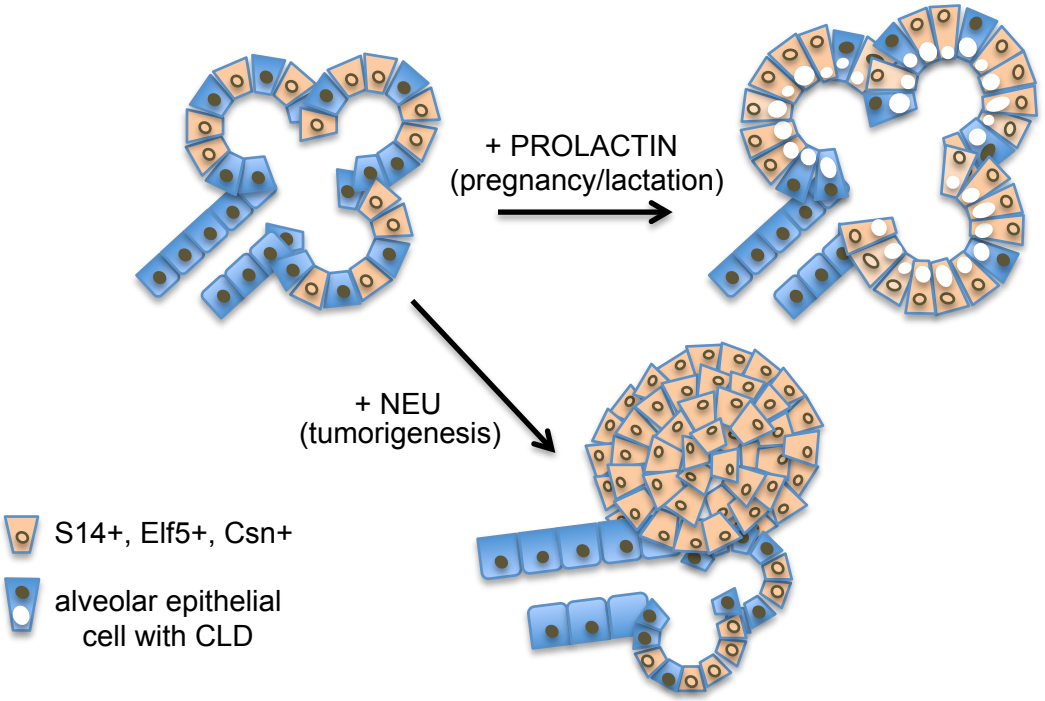



**Figure 11. Proposed model of Spot14 action in the mammary gland and breast cancer.** Based on our studies, we predict that Spot14 is expressed in a differentiated cell type within the mammary gland; a cell that also expresses Elf5 and Csn genes. In these cells, Spot14 elevates fatty acid synthesis. Under normal developmental circumstances, particularly during pregnancy and lactation, the mammary tissue is exposed to prolactin, which instructs the cells to produce milk from available fatty acids. In the context of oncogenic signaling, however, we expect that the Spot14-mediated increase in fatty acid synthesis gives these differentiated cells a proliferative advantage, which causes the early emergence of tumors. Because of their gene expression profiles, we predict that these tumors are not highly metastatic.

Key Research Accomplishments

- We have generated MMTV-Neu/MMTV-Spot14 (Neu/S14) mice and have completed the study on mammary tumorigenesis. This data is being prepared for submission to Cancer Research this month
- From the above model, we have demonstrated a role for Spot14 in stimulating the formation of Neu-induced mammary tumors and enhancing cell proliferation within those tumors.
- We have performed NMR, GC-mass spec, and microarray analyses on tumors from experimental and control groups and have shown that Neu/S14 tumors are well-differentiated, as they display hallmarks of the lactating mammary gland.
- We have shown that Neu/S14 tumors have elevated fatty acid contents, but that this is not sufficient to increas metastasis as we had previously suspected.
- I have learned to perform bioinformatics analysis of public human tumor datasets and metadata and have used these tools to support the studies performed in our mouse model.
- We have generated MMTV-PyMT/Spot14-null mice to satisfy the remaining part of this proposal and have performed initial analyses on these mice

Reportable Outcomes

A manuscript is in preparation describing these studies. We anticipate submitting this work to Cancer Research this month.

The results of these studies were presented at the Endocrine Society's annual meeting in Houston TX, in August 2012.

We have generated MMTV-Neu/MMTV-Spot14 bitransgenic mice that can be made available to other researchers.

From this tumor study, we have performed microarray analysis of tissues from Neu and Neu/S14 mice. These microarray data will be deposited in the NCBI Gene Expression Omnibus (GEO) database for access by the community.

Based on these studies, our laboratory has acquired transgenic mice in which expression of Fatty Acid Synthase (FASN) can be eliminated in developing tumors. The studies described here were used as background in an application for the DoD BCRP Idea Award mechanism this year, in which we proposed to perform tumor studies in mice with and without the ability to synthesize fatty acids de novo in cancer cells.

## Conclusions

Based on what we knew about the role of S14 in regulating de novo fatty acid synthesis and on its published role in breast cancer outcome, we hypothesized that S14 overexpression would elevate fatty acid synthesis in cancer cells, which would stimulate tumor formation, tumor growth, and tumor metastasis. To test this hypothesis, we generated a transgenic mouse model that expresses S14 in the mammary epithelium and crossed that model with an existing model of mammary tumorigenesis, the MMTV-Neu mouse. We have shown that S14 overexpression shortens tumor latency, stimulates proliferation of tumor cells and non-tumor epithelial cells, yet does not promote tumor metastasis. Using gene expression profiling, we identified that Neu/S14 tumors were more differentiated than Neu tumors, which explains their decreased metastatic activity. We also analyzed human breast tumors for S14 expression and associated high levels of S14 with a favorable outcome. This is a clear discrepancy with what was published for S14, however our analysis included several hundred primary breast tumors from multiple datasets.

## Why does this matter?

The conclusions of our studies are that elevated de novo fatty acid synthesis, per se, may be sufficient to stimulate cell proliferation and tumor growth, but does not appear to be sufficient for tumor metastasis. Rather, it appears that the metastatic ability of a tumor is hard-wired into its gene expression profile, and may not be so easily influenced by changes in tumor metabolism. This conclusion is supported by public human breast tumor microarray data, which shows that high S14 expression is protective from disease metastasis and from death due to disease. These studies suggest that S14 may be an important marker of differentiation status of human breast tumors that could give insight into the type of outcome a patient might experience.

References

1. Tusher VG, Tibshirani R, Chu G. Significance analysis of microarrays applied to the ionizing radiation response. Proc Natl Acad Sci U S A 2001;98(9): 5116-21.

2. Choi YS, Chakrabarti R, Escamilla-Hernandez R, Sinha S. Elf5 conditional knockout mice reveal its role as a master regulator in mammary alveolar development: failure of Stat5 activation and functional differentiation in the absence of Elf5. Dev Biol 2009;329(2): 227-41.

3. Hilton HN, Kalyuga M, Cowley MJ, et al. The antiproliferative effects of progestins in T47D breast cancer cells are tempered by progestin induction of the ETS transcription factor Elf5. Mol Endocrinol 2010;24(7): 1380-92.

4. Wells WA, Schwartz GN, Morganelli PM, Cole BF, Gibson JJ, Kinlaw WB. Expression of "Spot 14" (THRSP) predicts disease free survival in invasive breast cancer: immunohistochemical analysis of a new molecular marker. Breast Cancer Res Treat 2006;98(2): 231-40.

5. Rhodes DR, Yu J, Shanker K, et al. ONCOMINE: a cancer microarray database and integrated data-mining platform. Neoplasia 2004;6(1): 1-6.